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Structure of the Hairy-root-inducing Plasmid and Identification of its Replicator Region

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The causative agents of the hairy root disease in many dicotyledonous plants are the root-inducing plasmids carried by *Agrobacterium rhizogenes*. One of such plasmids, pRiA4b, was studied by constructing a gene library with cosmid vectors and by mapping HindIII restriction fragments. From this library, a mini-pRiA4b replicon of 4.6 kb[¶] was isolated, which was stably maintained in *Agrobacterium* spp. and had replication characteristics similar to those of the parental pRiA4b. Mini-pRiA4b derivatives would be useful as shuttle vectors between *Escherichia coli* and *Agrobacterium* spp. for genetic engineering experiments.

KEY WORDS: Ri plasmid/ Ti plasmid/ Gene library/ Restriction map/
Replication origin/ Shuttle vector/

INTRODUCTION

Hairy root and the related disease, crown gall, are caused by *Agrobacterium rhizogenes* and *A. tumefaciens*, respectively.¹⁾ These bacteria possess large plasmids called Ri (root-inducing) and Ti (tumor-inducing) plasmids.^{2,3)} Portions of plasmid DNA are transferred into plant cells and integrated into their nuclear genome.⁴⁻⁷⁾ The transferred DNA (T-DNA) encodes genes which control tumor morphology.^{8,9)} The T-DNA also encodes genes which direct the synthesis of unique amino acid derivatives called opines, such as octopine (*ocs*), nopaline (*nos*), agropine (*ags*), and agrocinopine (*acs*).^{10,11)} Another set of plasmid genes (*vir*), which is essential for virulence, is located outside the T-DNA.^{12,13)} Although the transformation events induced with Ri and Ti plasmids seem to be similar, homology of nucleotide sequences between these two plasmids is restricted to respective specific parts.¹⁴⁾ In addition, Ri and Ti plasmids belong to different incompatibility groups and they can be maintained stably together in one cell.¹⁵⁾ The replication and incompatibility region (replicator region) of the octopine Ti plasmid (pTiB6) has been localized¹⁶⁾ and is homologous to the corresponding region of nopaline Ti plasmid (pTiC58).¹⁷⁾

To assist in our understanding of the molecular basis of the characteristics of these plasmids and in constructing useful vectors for plant gene transfer, we have now constructed a gene library of the agropine Ri plasmid, pRiA4b. We have also isolated from the library mini-Ri replicons which are stably maintained in *Agrobacterium* cells, and the replicator region has been precisely mapped.

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¶ Abbreviation used: kb, kilobase-pairs; Ap, ampicillin; Cb, carbenicillin; Km, kanamycin; suffix r, resistance or resistant to drug; Tc, tetracycline.

MATERIALS AND METHODS

(a) General methods

Methods for transformation of *E. coli*, preparation of plasmid DNA from *E. coli* cells, restriction endonuclease digestion, repair synthesis, ligation, gel electrophoresis with agarose and polyacrylamide, extraction of DNA fragments from gels, rapid clone analysis, and radioactive labeling by nick translation have been described previously.¹⁸⁾ Southern transfer and hybridization were performed as reported.¹⁹⁾

(b) Culture media and antibiotics

L broth (10 g of polypeptone, 5 g of yeast extract, 5 g of NaCl and 1 g of glucose/l; pH 7.2) was used for cultivation of *E. coli* strains. The culture media used for *Agrobacterium* strains were peptone medium (4 g of peptone/l of 2 mM MgSO₄; pH 7.2) and YEB medium (5 g of beef extract, 1 g of yeast extract, 5 g of peptone, 5 g of sucrose/l of 2 mM MgSO₄; pH 7.2). L agar and YEB agar both contained 1.5% agar. Antibiotics were used in the following concentrations: ampicillin, 20 µg/ml; kanamycin, 20 µg/ml; carbenicillin, 170 µg/ml; rifampicin, 50 µg/ml; tetracycline, 7 µg/ml; chloramphenicol, 50 µg/ml.

(c) Bacterial and plasmid strains

E. coli strains used were DH1 (F⁻ *recA1 endA1 gyrA96 thi1 hsdR17 supE44 relA1*) and JM109 (*recA1 endA1 gyrA96 thi1 hsdR17 supE44 relA1 Δ(lac~proAB)/F'* carrying *traD36 proA⁺B⁺ lacI^s lacZ ΔM15*).²⁰⁾ The *hsdR17* mutation is restriction (K)⁻ and modification (K)⁺. *Agrobacterium rhizogenes* A4 was a gift from Dr. C. Matsui (Nagoya University). *Agrobacterium tumefaciens* strains used were: R1000 (pRiA4b),¹⁴⁾ a transconjugant of cured C58 and *A. rhizogenes* A4; GV3101,²¹⁾ a rifampicin^r derivative cured of pTiC58; C58-C1 Cm^r (pTiC58*tra^c*)²¹⁾; and C58-C1 Cm^r (pTiB6S3*tra^c*).²²⁾

Four plasmid vectors derived from ColE1 were pHC79 (*cos bla*),²³⁾ pUC18 (*bla*),²⁰⁾ pAO213 (*bla kan cos oriT*) and pAO264 (*kan*). pAO213 was constructed by combining the larger BamHI-HindIII fragment (6.1 kb) of pHC79 (*bla cos oriV*), the *kan* gene of Tn5 (1.8 kb BamHI-HindIII fragment) and the transfer origin of RK2 (0.76 kb-BamHI fragment of pEYDG1²⁴⁾). pAO264 that was similar to pUC18 but carried *kan* instead of *bla*, was made from 1/4ColE1 (pAO3), HindIII-SmaI fragment of Tn5 and HaeII fragment carrying the multiple cloning site within *lacZ* of pUC18. pRK212.1 (*tet bla*) is a self-transmissible Km^s derivative of RK2,²⁵⁾ and pVK100 (*tet kan ori cos*) is a small non-self-transmissible RK2 derivative.²⁶⁾

(d) Preparative plasmid isolation from *Agrobacterium*

Agrobacterium cells carrying plasmids were fully grown in 1 l of peptone medium, and cells were collected by centrifugation (8,000 rpm, 10 min, 4°C), washed with 100 ml of 20 mM EDTA-50 mM Tris.HCl (pH 8.0), suspended in 80 ml of the same buffer, and then mixed with 10 ml of pronase solution (10 mg/ml) and 10 ml of 10% sodium dodecylsulfate. The mixture was incubated at 37° for 2 h to allow complete lysis, and then vigorously stirred by a giant magnetic stirrer in order to lower its viscosity. Alkali (3 N NaOH) was added drop by drop to the lysate with gentle stirring until a pH of 12.4 was reached. After 15 min, the mixture was neutralized

by adding 2 M Tris.HCl (pH 7.0) followed by the addition of 5 M NaCl to a final concentration of 1 M, and then kept at 4°C for 4 h. Cleared lysate obtained by centrifugation (20,000 rpm, 20 min, 4°C) was mixed with 50% polyethylene glycol (#6000) to a final concentration of 10%, and kept at 0°C for 15 h. Precipitates produced were collected by centrifugation (8,000 rpm, 15 min, 4°C), dissolved in 5 ml of 20 mM EDTA-50 mM Tris.HCl (pH 7.5). Insoluble materials were removed by centrifugation (20,000 rpm, 20 min, 4°C). The DNA solution thus obtained was subjected to ethidium bromide-CsCl density gradient centrifugation, and covalently closed circular DNA molecules were isolated. Dye was removed by n-butanol extraction.

(e) *Rapid procedure for detection of Agrobacterium plasmids*

Agrobacterium cells grown in 1 ml of YEB medium were collected by centrifugation in an Eppendorf tube, suspended in 0.2 ml of 2 mM EDTA-40 mM Tris.CH₃-COOH (pH 7.9) and mixed with 0.4 ml of 3% sodium dodecylsulfate-50 mM Tris. NaOH (pH 12.6). After heating the mixture at 65°C for 20 min, proteins and cell debris were removed by brief extraction with unbuffered phenol-chloroform. The clarified extract (pH about 9) was used directly for gel electrophoretic analysis. These plasmid preparations could be stably maintained at 4°C for several weeks.

(f) *Transformation of Agrobacterium*

The freeze-thaw method described earlier²⁷⁾ was modified. A preculture of recipient bacteria was grown overnight at 28°C in YEB medium and transferred to 250 ml of fresh YEB medium at a cell density of 5×10^7 cells/ml. This culture was further incubated to reach a cell density of 5×10^8 cells/ml, and cells were spun down by centrifugation (7,000 rpm, 10 min, 4°C), washed with 50 ml of 10 mM Tris.HCl (pH 7.5) and then suspended in YEB medium at about 5×10^{10} cells/ml. The concentrated bacterial suspension (0.2 ml) was mixed with 0.1 ml of a DNA dilution (about 20 µg/ml of YEB medium). After quickly freezing the mixture in liquid nitrogen for 5 min, it was thawed at 37°C and then kept for 25 min at this temperature. The sample was mixed with 1.2 ml of fresh YEB medium, incubated at 28°C for 12 h to allow phenotypic expression and spread on appropriate agar plates to determine both the numbers of total bacteria as well as of transformants. To verify the competence of recipient cells, transformation with pVK100 was always performed as a standard.

(g) *Tri-parental conjugation*

Bacterial strains used for mating were GV3101, DH1(pRK212.1) and DH1 carrying one of pBANK cosmids (see RESULTS). The three bacterial cultures (1.5 ml each) grown to 5×10^8 cells/ml in YEB medium were mixed and passed through a 0.45 µm-Millipore filter. The filter was incubated at 28°C overnight on a non-selective YEB agar plate. The cells were resuspended and plated for transconjugants on appropriate selective agar plates.

(h) *Packaging of cosmids in lambda phage particles in vitro*

Two packaging mixes were prepared by a procedure of Grosveld *et al.*²⁸⁾ using two lambda-lysogenic *E. coli* strains, BHB2688 and BHB2690.

RESULTS AND DISCUSSION

(a) Cloning and *Hind*III restriction mapping of *pRiA4b* DNA

Agrobacterium rhizogenes A4 contained three plasmids, *pRiA4a* (ca. 170 kb), *pRiA4b* (ca. 250 kb) and *pRiA4c* (ca. 420 kb). *pRiA4c* was not able to be maintained with either *pRiA4a* or *pRiA4b* in a single bacterium and its size roughly corresponded to the sum of the other two. Upon cultivation of *pRiA4c*-carrying cells, it was found that both *pRiA4a* and *pRiA4b* appeared at an equal frequency. Therefore, *pRiA4c* was concluded to be a cointegrate between *pRiA4a* and *pRiA4b*. This situation is similar to another Ri plasmid *pRi15834*.²⁹⁾ Huffman *et al*¹⁴⁾ have isolated a bacterial strain carrying only one species (*pRiA4b*) of the above three plasmids, which is still capable of root-inducing as well as the parental A4 strain. This bacterial strain was used for the preparation of *pRiA4b* plasmid DNA in the present work. *pRiA4b* created 48 fragments with sizes greater than 1.0 kb upon digestion with *Hind*III. For the construction of a gene library of *pRiA4b*, plasmid DNA was partially digested with *Hind*III and fragments of 38–43 kb were isolated by a sucrose-density gradient centrifugation followed by insertion into the unique *Hind*III site of one of two cosmid vectors. The smaller one (*pHC79*)²³⁾ enabled us to clone larger segments and the other one (*pAO213*) which carried the transfer origin of the broad host range plasmid

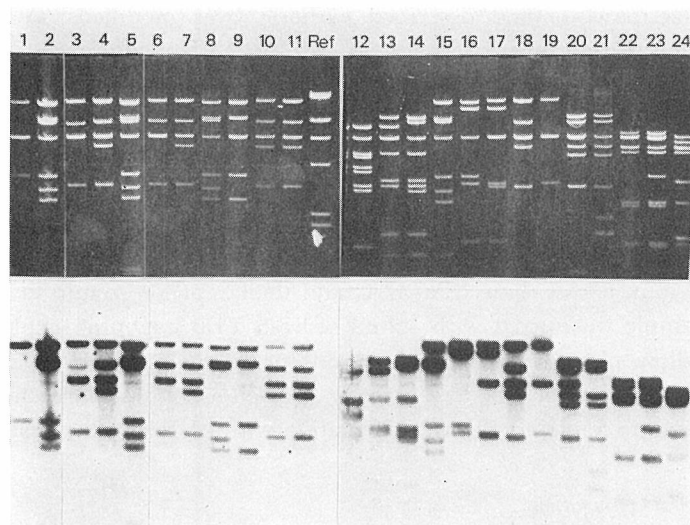


Fig. 1 *pBANK* cosmid DNA was digested with *Hind*III and the resulting DNA fragments were separated by 0.7% agarose gel electrophoresis. After staining the gel with ethidium bromide, a photograph was taken under ultraviolet light (Upper). The DNA fragments were transferred to a nitrocellulose filter and then an autoradiograph was taken (Lower). Cosmids analyzed were *pBANK102* (1), *pBANK103* (2), *pBANK106* (3), *pBANK109* (4), *pBANK114* (5), *pBANK204* (6), *pBANK215* (7), *pBANK141* (8), *pBANK145* (9), *pBANK213* (10), *pBANK214* (11), *pBANK216* (12), *pBANK210* (13), *pBANK209* (14), *pBANK128* (15), *pBANK130* (16), *pBANK219* (17), *pBANK101* (18), *pBANK138* (19), *pBANK121* (20), *pBANK205* (21), *pBANK206* (22), *pBANK207* (23) and *pBANK330* (24). Ref is *Hind*III digest of lambda DNA.

RK2,²⁵⁾ would permit transfer of cloned segments to *Agrobacterium* spp. with the assistance of an appropriate helper plasmid. By using the lambda packaging system *in vitro*,²⁸⁾ recombinant cosmids were introduced into *E. coli* DH1 cells, and many Ap^r transductant colonies were obtained. Plasmid DNA carried by respective transductants were isolated and characterized by HindIII digestion. These hybrid cosmids were named pBANK000 for the pHc79 vector series and pBANK0000 for the pAO213 vector series. As expected, each clone contained various HindIII fragments of pRiA4b. The fidelity of each clone was checked by comparing the restriction fragments of pBANK against the restriction fragments of pRiA4b generated by HindIII and also by Southern hybridization with the probe of ³²P-pRiA4b. Some representative HindIII restriction patterns of pBANK cosmids and their Southern hybridization data are presented in Fig. 1. A series of overlapping clones permitted us to deduce the fragment order. Although there is no overlapping clone across the junction between fragments 23b and 24c, their contiguity was verified from the HindIII restriction pattern of EcoRI fragment 6 of pRiA4b covering this region. The deduced HindIII map and the DNA regions carried by each pBANK clone are shown in Fig. 2. This map is essentially the same as one which was reported previously.¹⁴⁾ The gene library and the HindIII map of pRiA4b constructed here would contribute to *in vitro* genetics of various functional regions, in relation to the well-examined Ti plasmids.

(b) *Identification of the replicator region*

A set of 20 pBANK cosmids constructed with the pAO213 vector, which covered the entire pRiA4b, was first transferred from *E. coli* to an *Agrobacterium* strain cured of its Ti plasmid (GV3101) in the presence of helper plasmid pRK212.¹²⁵⁾ by tri-parental conjugation experiments. The pBANK cosmids used were pBANK1105, pBANK1108, pBANK1123, pBANK1129, pBANK1145, pBANK1302, pBANK1303, pBANK1313, pBANK1314, pBANK1317, pBANK1329, pBANK1362, pBANK1367, pBANK1373, pBANK1375, pBANK1380, pBANK1385, pBANK1386, pBANK1393 and pBANK1395. Only two of these cosmids, pBANK1108 and pBANK1129, transferred its Km^r marker to the recipient cells at high efficiencies. However, other pBANK cosmids could also transfer the Km^r marker at lower but considerable frequencies. Since the former transfer but not the latter transfer was able to occur without the simultaneous transfer of unselected Tc^r marker carried by the helper plasmid, these observations were interpreted as that pBANK1108 and pBANK1129 were able to be maintained as a single replicon in GV3101 and the other pBANK cosmids could only replicate as cointegrates with the helper plasmid in GV3101.

To confirm this explanation, another set of 17 pBANK cosmids pBANK121, pBANK130, pBANK147, pBANK155, pBANK205, pBANK206, pBANK209, pBANK211, pBANK216, pBANK219, pBANK330, pBANK1108, pBANK1129, pBANK1303, pBANK1329, pBANK1362, and pBANK1385 were used to transform GV3101. Again, pBANK1108 and pBANK1129, and two other cosmids pBANK121 and pBANK205 gave Km^r and Cb^r transformants, respectively, at efficiencies of 10⁻⁷ to 10⁻⁸ per recipient cell under the conditions used. Covalently closed circular DNA molecules were able to be isolated from these transformant cells and their yield cor-

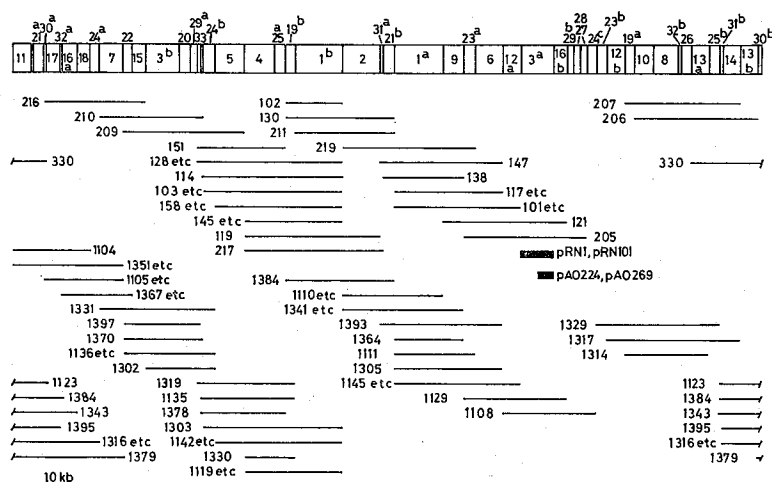


Fig. 2 HindIII restriction map of pRiA4b is at the top labeled with their corresponding fragment number. The circular pRiA4b DNA is illustrated as a linear molecule by cutting at the junction between HindIII fragments 11 and 30b. DNA regions carried by each pBANK cosmid are shown by a horizontal line with the pBANK number. Thick bars represent DNA regions carried by mini-pRiA4b replicons derived from pBANK205. In addition to the drawn clones, there were: pBANK-109, pBANK129, pBANK142, pBANK212, pBANK213, pBANK214, pBANK215 and pBANK325 same as pBANK101; pBANK204 same as pBANK117; pBANK-139, pBANK141 and pBANK208 same as pBANK103; pBANK155 same as pBANK-128; pBANK221 and pBANK316 same as pBANK158; pBANK311 same as pBANK-145; pBANK1373 same as pBANK1351; pBANK1133 and pBANK1318 same as pBANK1105; pBANK1388 same as pBANK1367; pBANK1202, pBANK1327 and pBANK1380 same as pBANK1136; pBANK1311, pBANK1322, pBANK1324, pBANK1344, pBANK1352, pBANK1359 and pBANK1365 same as pBANK1142; pBANK1121, pBANK1332 and pBANK1333 same as pBANK1119; pBANK1348 and pBANK1390 same as pBANK1110; pBANK1362 same as pBANK1341; pBANK1313 same as pBANK1145; and pBANK1387 same as pBANK1316. "1384" carrying the Hind III fragment 1b should be read "1386".

responded to a copy number per cell of about 2. Their sizes and restriction patterns were indistinguishable from those of the donor DNA used for transformation. Therefore, it was concluded that these four pBANK cosmids were capable of replicating in *Agrobacterium* cells and that their common region (HindIII fragments 12a, 3a and 16b) contained the replication origin of pRiA4b. It seems unlikely that a replication origin other than this is present.

In order to define more narrowly the region needed for autonomous replication in *Agrobacterium* cells, pBANK205 DNA was cut with HindIII and each fragment was independently subcloned in pUC18 (Cbr^r) and pAO213 (Km^r). Only the plasmids containing HindIII 3a fragment (pRN1 and pRN101, respectively) were stably maintained in GV3101, indicating that the pRiA4b replicative functions are all contained within this fragment. It has previously been reported that this fragment is highly homologous to the replication region of octopine-type plasmid pTiA6.¹⁴ Since the HindIII 3a fragment carried a single BamHI susceptible site near its center, two BamHI-HindIII subfragments were cloned in pUC18. Of the two constructions,

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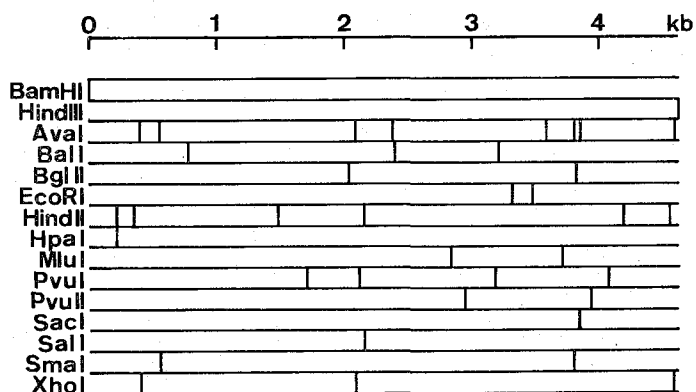


Fig. 3 Restriction maps of the 4.6 kb replicator fragment of pRiA4b. Each cleavage site is shown by a vertical line.

only pAO224 carrying the smaller BamHI-HindIII fragment (4.6 kb) confers Cb^r on its host bacterium. When the Km^r pAO264 vector was used instead of pUC18, similar results were obtained (a replicative recombinant plasmid was named pAO269). Several restriction enzymes were used to map this 4.6 kb region (Fig. 3). Subcloning experiments of the 4.6 kb fragment, using enzymes generating smaller fragments, were performed. Two SalI subfragments (2.2 kb and 2.4 kb), SmaI fragment (3.25 kb) and two XhoI fragments (1.69 kb and 2.51 kb) all were unable to replicate. Elimination of a small EcoRI fragment (0.15 kb) from the 4.6 kb fragment resulted in the loss of replicating ability. These results suggest that almost the entire region of 4.6 kb fragment is required for autonomous replication. An insertion of a tiny segment but not a long fragment at the SalI site was not detrimental to replication function. This result suggests the presence of spacer sequences in the pRiA4b origin similar to those identified in *E. coli oriC* which keep multiple binding sites of replication initiation proteins at proper distances.¹⁸⁾ The entire nucleotide sequence of the 4.6 kb segment has recently been determined which will appear elsewhere (Nishiguchi, Takanami and Oka).

(c) Characteristics of mini-pRiA4b plasmids

Stability of mini-pRiA4b plasmids constructed above were examined by grow-

Table 1.

Hybrid plasmid (Vector; Marker)	pBANK1108 (pAO213; Km)	pBANK121 (pHC79; Cb)	pRN1 (pUC18; Cb)	pRN101 (pAO213; Km)	pAO224 (pUC18; Cb)
Curing frequency	<0.2%	<0.2%	0.4%	0.3%	2%

GV3101 cells carrying each of five plasmids which were purified on a selective agar plate was transferred to YEB medium at 10^5 cells/ml and then grown to 3×10^9 cells/ml (15 generations). Cells were spread for about 600 colonies on a YEB agar at 28°C, and colonies produced were replicated to an appropriate selective agar at 28°C. The ratio of the number of drug sensitive colonies to that of the total colonies were expressed as curing frequency. These values were averages of three separate experiments. DNA regions carried by five hybrid plasmids are shown in Fig. 2.

ing the host GV3101 cells in the absence of selective pressure at 28°C. The results summarized in Table 1 show that each of the five mini-pRiA4b plasmids are fairly stable in *A. tumefaciens*, since they were lost with a frequency lower than 3% during 15 generations under the conditions used. However, the smaller the pRiA4b origin sequences were, the less stable they became in GV3101 cells. This might be due to the existence of stability gene(s) outside the 4.6 kb segment. In contrast to the situation at 28°C, these plasmids were easily curable by growing the host cells at higher temperature. For instance, when GV3101 carrying pBANK1108 or pRN101 was grown for 15 generations at 35°C, 70 to 85% cells were plasmid-free.

To test the incompatibility of these mini-pRiA4b, pBANK1108 was used to transform three *A. tumefaciens* strains each carrying pTiC58 tra^c , pTiB6S3 tra^c or pRiA4b. The presence of plasmids was verified by the rapid method of plasmid isolation. It was found that pBANK1108 expressed incompatibility toward pRiA4b but not toward pTiC58 and pTiB6S3. All these replicative characteristics of mini-pRiA4b were the same as those of the parental pRiA4b.

The mini-pRiA4b replicons described in this paper carried the amplifiable ColEI origin, and were thus able to replicate in both *Agrobacterium* spp. and *E. coli*, making these hybrid plasmids potentially useful as shuttle vectors. Although Close *et al.*³⁰⁾ also constructed shuttle vectors derived from pBR322 and pSa, they are less stable in *A. tumefaciens* than the shuttle plasmids described here. A better knowledge of the replication origins of plasmids of *A. rhizogenes* strain A4 and the construction of these new vector candidates is the first step in the construction of binary systems with *A. rhizogenes* plasmids for plant genetic engineering experiments. During the preparation of this manuscript, a similar mini-pRiA4b derived from BamHI 11 fragment has been reported.³¹⁾

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